Fundamentals of Clinical Chemistry

THIRD EDITION

EDITOR

Norbert W. Tietz, Ph.D.

Professor, Department of Pathology,
Director of Clinical Chemistry,
College of Medicine,
University of Kentucky,
Lexington, Kentucky

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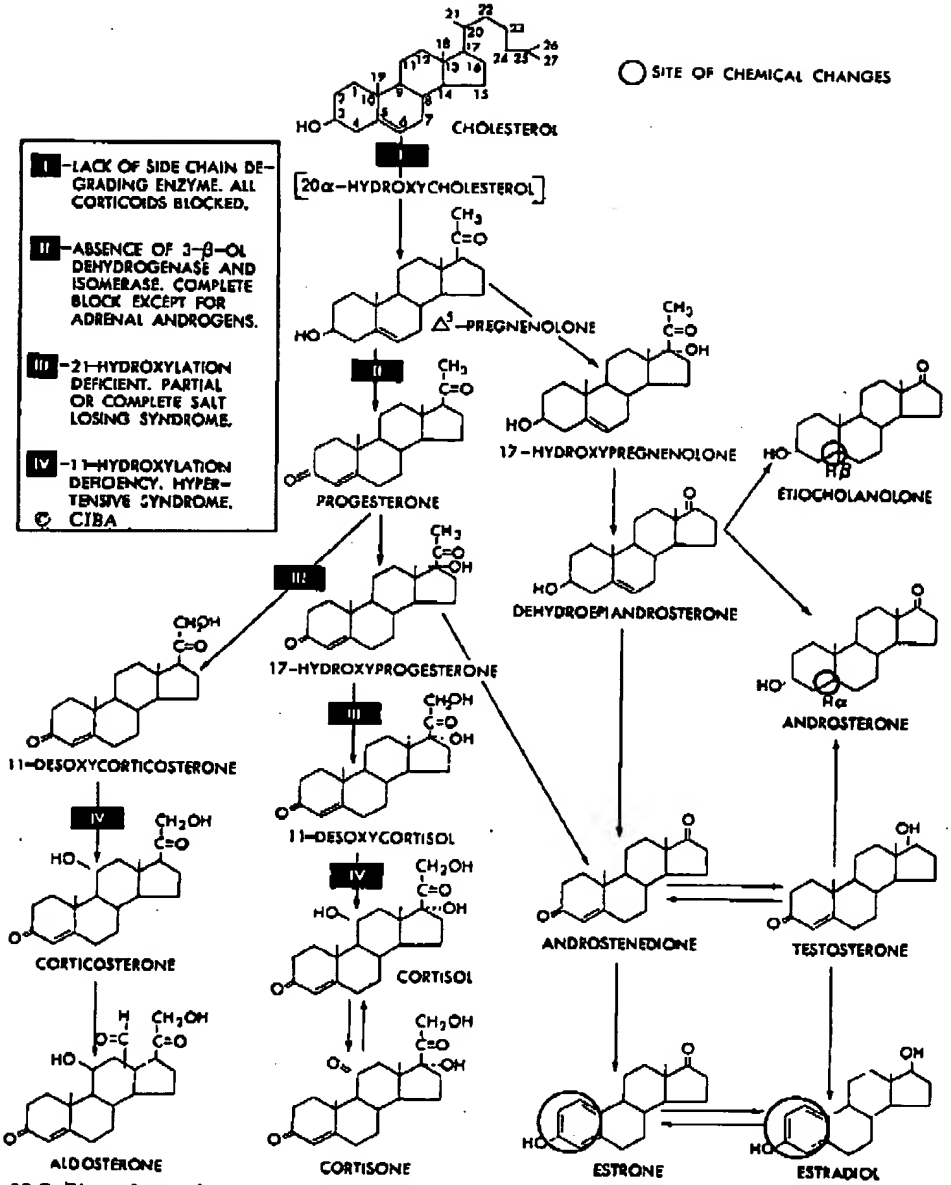

Figure 18-3. Biosynthesis of corticosteroids. I, II, III, and IV indicate sites of major blocks causing adrenogenital syndromes. (Modified from *The CIBA Collection of Medical Illustrations* by Frank H. Netter, M.D. © Copyright 1959 CIBA Pharmaceutical Company, Division of CIBA-GEIGY Corporation.)

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fraction is applied to the column, and the column is washed with phosphate-buffered saline. Additional washing with a solution of 0.125 *M* borate, pH 8.5, 1.0 *M* NaCl, 0.1% Tween 20 removes nonspecifically bound protein.[55] The antibodies are eluted with 2 *M* acetic acid in the cold and neutralized immediately.

The immunospecifically purified antibodies have been particularly useful in physicochemical studies of the antibody–nucleic acid interaction; for some purposes, the monovalent Fab fragments of the purified antibodies have been used.[54,56] The use of absorbed and purified antibodies also ensures the specificity of immunofluorescent and serological measurements of helical nucleic acids of a given class in the presence of other nucleic acid forms.

Acknowledgments

Research in the author's laboratory has been supported by grants (currently grant PCM-79-04057) from the National Science Foundation and grant AI14534 from the National Institutes of Health.

[55] J. A. Smith, J. G. R. Hurrell and S. J. Leach, *Anal. Biochem.* **87**, 299 (1978).
[56] M. Leng, M. Guigues, and D. Genest, *Biochemistry* **17**, 3215 (1978).

[4] The Preparation of Antigenic Hapten–Carrier Conjugates: A Survey

By BERNARD F. ERLANGER

Substances of molecular weight less than 1000 are not ordinarily antigenic. However, antibodies can be raised to small molecules by immunization with conjugates made up of low molecular weight substances (haptens) covalently linked to proteins or synthetic polypeptides. The ability to couple many different structures to macromolecules, the high degree of antigenicity of many of the conjugates, the development of sensitive methods of detecting and quantitating reactions between antibody and hapten, and the perfection of techniques for obtaining highly purified preparations of antihapten antibodies have contributed to the development of many of our modern immunological concepts. Much of our current knowledge of the requirements for immunogenicity, the structure of antigenic determinants, and the nature of antibody—its purification, het-

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erogeneity, valence, size of combining site, and biological properties—has resulted from the use of such conjugates.

The immunochemistry of low molecular weight molecules had its beginning in the pioneering work of Landsteiner. In 1917, when Landsteiner set out to prepare what he called "artificial conjugated antigens," it was "to investigate an almost dogmatic belief . . . that a special chemical constitution, peculiar to proteins, was required for the production of antibodies."[1] We know better now, of course, but it is to these studies by Landsteiner that we owe much of what appears in this volume.

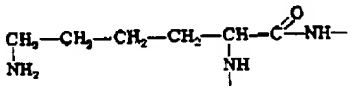
The earliest of his conjugated proteins were prepared by the acylation of the amino groups of serum albumin with chlorides or anhydrides of butyric, isobutyric, mono-, di-, and trichloroacetic, anisic, and cinnamic acids. This was followed by his better-known studies in which diazonium compounds were allowed to react with histidine, tyrosine, and tryptophan residues of a protein. With these conjugates, he established that the original specificity of the protein carrier was changed by the newly introduced groups which, by themselves, were not antigenic, and that cross-reactions among sera depended now upon the structural relationships among the acyl or azo groups that were covalently linked to the protein. He also noted that, in most cases, antibody was produced to the protein carrier as well and, to be certain of antibodies to the new determinant group, one had to test the sera with conjugates made with an unrelated or homologous (to the immunized animal) protein. It was his practice to remove any of the anticarrier antibody by absorption of the serum with the free protein. This is still done, although it is not necessary for radioimmunoassays. Landsteiner also sought to determine the optimal number of haptenic groups that gave the best antibody response, and he concluded that too much or too little hapten led to a poor response. With serum albumin as the carrier, 10 haptenic groups seemed to be optimal. The major finding by Landsteiner, however, related to the exquisite specificity of the antisera, as was so beautifully demonstrated by his classical studies with L-, D-, and *meso*-tartaric acids.

Thus, Landsteiner's work established many of the ground rules by which we operate today. Our contributions since his time have been mainly refinement of techniques and procedures and the expansion of his ideas. The major exception to this statement, and a crucial one indeed, is the development by Berson and Yalow[2] of the technique of radioimmuno-

[1] K. Landsteiner, "The Specificity of Serological Reactions" Harvard Univ. Press, Cambridge, Massachusetts, 1945.

[2] S. A. Berson and R. S. Yalow, Radioimmunoassay: A status report, *in* "Immunobiology: Current Knowledge of Basic Concepts in Immunology and Their Clinical Application" (R. A. Good and D. W. Fisher, eds.), pp. 287–293. Sinauer, Stamford, Connecticut, 1971.

ε-AMINO GROUPS OF LYSINE RESIDUES *(59)*



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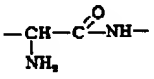
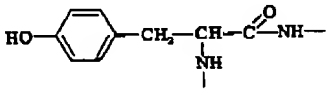
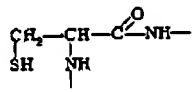


PHENOLIC HYDROXYL GROUPS OF TYROSINE RESIDUES *(19)*



SULFHYDRYL GROUPS OF CYSTEINE RESIDUES *(1)*



IMIDAZOLE GROUPS OF HISTIDINE RESIDUES *(17)*

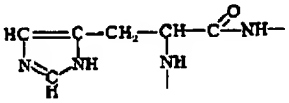


FIG. 1. Available functional groups in bovine serum albumin.

assay. It is this procedure that has led to the dramatic expansion of immunological techniques into the fields of biochemistry and pharmacology.

In 1956 our laboratory, in collaboration with Beiser and Lieberman, became interested in preparing steroid–protein conjugates that were to be used to elicit antisteroid antibodies. An examination of the literature at that time showed that the azo coupling techniques of Landsteiner were still dominant. Like him, we chose to use the serum albumins because they were inexpensive and likely to yield soluble conjugates. However, an examination of the amino acid content of bovine serum albumin (BSA) (Fig. 1) convinced us that substitution by such relatively complex haptens as steriods should be attempted by reaction with the more plentiful ε-amino groups of the lysine residues rather than by an azo coupling reaction with tyrosine, tryptophan, and imidazole residues. This meant forma-

tion of amide bonds, for which a number of convenient new methods had been developed for the synthesis of peptides.[3] A systematic approach was developed in which carboxylic acid groups were introduced into the haptens in various ways so that reaction with the amino groups of the protein carrier could be effected.

Rather than deal with the steroid work separately, we will incorporate it into a general survey of the methods of preparing immunogenic hapten–protein conjugates in which the hapten is a pharmacologically interesting compound. The arrangement will be governed by the nature of the reactive functional groups of the hapten. In this way, the information can be applied most easily to new compounds being considered for use as determinant groups. No attempt will be made to present an exhaustive review of the literature. Instead, the various procedures described will be illustrated by specific examples to which the reader can refer for practical aspects of the experimental methods.

Choice of Carrier

The protein carriers used in various laboratories include globulin fractions, the serum albumins of various species, hemocyanin, ovalbumin, thyroglobulin, and fibrinogen. Hapten–protein conjugates of serum albumin are, in general, more soluble than conjugates of γ-globulin or of ovalbumin. Thus, for example, steroid–protein conjugates of bovine, rabbit, and human serum albumin were soluble above pH 5.5[4,5]; similar conjugates made with γ-globulin and egg albumin frequently precipitated out of solution during preparation and could not be redissolved. Insoluble conjugates can be used for immunization, but subsequent characterization of the antibody then becomes a more difficult problem.

Under certain circumstances, it may be advantageous to have both soluble and insoluble conjugates containing the same determinant group. The latter can be used for the isolation and purification of hapten-specific antibody.[6] For a review of insoluble hapten–carrier conjugates, we refer the reader to Jakoby and Wilchek[7] and Williams and Chase.[8]

[3] J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," Vol.2. Wiley, New York, 1961.
[4] B. F. Erlanger, F. Borek, S. M. Beiser, and S. Lieberman, *J. Biol. Chem.* **228**, 713 (1957).
[5] B. F. Erlanger, F. Borek, S. M. Beiser, and S. Lieberman, *J. Biol. Chem.* **234**, 1090 (1959).
[6] H. Szafran, S. M. Beiser, and B. F. Erlanger, *J. Immunol.* **103**, 1157 (1969).
[7] W. B. Jakoby and M. Wilchek, eds., This series, Vol. 34.
[8] C. A. Williams and M. W. Chase, eds., "Methods in Immunology and Immunochemistry," p. 335 *et seq.* Academic Press, New York, 1967.

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Whether the choice of carrier significantly influences the antihapten response is a controversial subject. In the author's opinion, no really definitive study has been carried out. On the other hand, there are some who believe that KLH is a superior carrier.[9] Thyroglobulin is a choice of others.[10] As noted above, we chose the serum albumins, as have the great majority of the laboratories engaged in developing radioimmunoassays.

With respect to the advantage of using a protein rather than a synthetic polypeptide as a carrier, we can refer to Jaffe *et al.*,[11] who showed that an active fragment of gastrin, its C-terminal tetrapeptide amide, was antigenic when covalently attached to serum protein carriers but not when linked to poly(L-lysine) or to poly(L-glutamic acid). Walker *et al.*[12] in 1973 made similar comparisons with steroid conjugates and obtained the same result, i.e., bovine serum albumin was a better carrier than poly(L-lysine). (But also see below.[13,14])

Optimal Epitope Density

Another important question concerns the optimal number of haptens bound to the carrier protein (i.e., optimal epitope density). Niswender and Midgley,[13] using steroid protein conjugates, suggested that at least 20 molecules of hapten should be covalently attached to a BSA carrier. Less than that results in an inferior antigen. Klause and Cross[14] in studies on $(DNP)_nBSA$ conjugates obtained good responses with as few as five DNP groups, with excellent booster responses. Comparable responses were obtained with $(DNP)_{15}BSA$. On the other hand, $(DNP)_{30}BSA$ and $DNP)_{60}BSA$ elicited an IgM response only; no change (i.e., boost) in titer occurred after 21 days. even with repeated immunization. It has been our experience that the nature of the hapten exerts an influence, but that good antibody titers can usually be obtained with epitope densities anywhere between 8 and 25. On the other hand. we have not hesitated to immunize with conjugates with fewer haptenic groups (as few as two) if we were unable to prepare "better" conjugates (for example, with expensive oligonucleotide–protein conjugates). We have never failed to obtain a response, although on occasion we have had to wait longer for a suitable titer.

[9] M. B. Rittenberg and A. A. Amkraut, *J. Immunol.* 97, 421 (1966).
[10] F. Bartos, G. D. Olsen. R. N. Leger, and D. Bartos, *Res. Commun. Chem. Pathol. Pharmacol.* 16, 131 (1977).
[11] B. M. Jaffe, W. T. Newton. and J. E. McGuigan, *Immunochemistry.* 7, 715 (1970).
[12] C. S. Walker. S. J. Clark. and H. H. Wotiz, *Steroids* 21, 259 (1973).
[13] G. D. Niswender and A. R. Midgley, Jr., *in* "Immunological Methods in Steroid Determination" (F. G. Peron and B. F. Caldwell. eds.). Ch. 8. Appleton, New York, 1970.
[14] G. G. B. Klause and H. M. Cross. *Cell. Immunol.* 14, 226 (1974).

Location of the Linkage Site

Before we go on to describe the various chemical reactions used to prepare conjugates, another important consideration must be recognized: the point of attachment on the hapten. Landsteiner established in his early studies that antibody specificity is directed primarily at the part of the hapten molecule farthest removed from the functional group that is linked to the protein carrier.[1] Our experiences have been similar. For example, many steroids share a common ring A structure. In agreement with Landsteiner, it was found that anti-testosterone-3-BSA was better able to distinguish among closely related steroids than was anti-testosterone-17-BSA.[15–17] Other similar studies exist in the literature.[15,18] Even better specificity has been obtained with conjugates in which the attachment to the steroid is via a spacer joining a protein to a position on the haptenic molecule that is not important for its biological specificity, e.g., the C-6 position of an estrogen,[19–21] on the C-6 or C-11 of progesterone.[19] An excellent investigation on the effect of the site of conjugation of corticosteroids is that of Nishina *et al.*[22] The antibody raised to these conjugates is thus specific for all the important structural features of the hapten.

Preparation of the Conjugates

Regardless of the protein carrier used, the same functional groups are available for attachment to the hapten: the carboxyl groups of the C terminal and of the aspartic and glutamic acid residues, the amino groups of the N terminal and the lysine residues, the imidazo and phenolic functions of the histidine and tyrosine residues, respectively, and the sulfhydryl group of cysteine residues (Fig. 1). All have been used for the preparation of immunogenic hapten–protein conjugates. Theoretically, the guanidino group of arginine is also available, but, to our knowledge, it has not been utilized in the preparation of conjugates.

The functional groups of the hapten govern the selection of the method to be used to conjugate the hapten to the functional groups of the carrier. The procedures described below, therefore, have been classified accord-

[15] S. M. Beiser and B. F. Erlanger, *Nature (London)* **214**, 1044 (1967).
[16] S. M. Beiser, B. F. Erlanger, F. J. Agate, and S. Lieberman, *Science* **129**, 564 (1959).
[17] S. Lieberman, B. F. Erlanger, S. M. Beiser, and F. J. Agate, *Recent Prog. Horm. Res.* **15**, 165 (1959).
[18] J. E. Buster and G. E. Abraham, *Anal. Lett.* **6**, 147 (1973).
[19] H. R. Lindner, E. Peril, A. Friedlander, and A. Zeitlin, *Steroids* **19**, 357 (1972).
[20] D. Exley, M. W. Johnson, and P. D. G. Dean, *Steroids* **19**, 605 (1971).
[21] S. L. Jeffcoate and J. E. Searle, *Steroids* **19**, 181 (1972).
[22] T. Nishina, A. Tsuji, and D. Fukushima, *Steroids* **24**, 861 (1974).

ing to the functional group of the hapten utilized for conjugation. In this way, it is hoped that the information may be applied most easily to compounds being considered for use as determinant groups.

Haptens with Carboxyl Groups

This class of haptens includes those that have a carboxyl group, such as acetylsalicylic acid (aspirin) or the peptides angiotensin and bradykinin. In addition, many haptens, such as some steroids, may have reactive groups to which a carboxyl group can be attached as, for example, by reaction with succinic anhydride (see below). For conjugation to proteins, the same procedures may be used regardless of whether the carboxyl group is present as an inherent part of the hapten or as an added moiety.

Mixed Anhydride Procedure

This is a simple, direct procedure[3,23] that does not require the preparation and isolation of an active derivative. The coupling procedure is carried out directly with the hapten, and the product usually contains 15–25 hapten groups per molecule of albumin. As an example of this method, the coupling of cortisone-21-hemisuccinate to protein[4] is illustrated (Fig. 2). The haptenic group was converted *in situ* to an acid anhydride, which could then react in an aqueous-acetone solution with the amino groups of serum albumin.

Uridine 5′-carboxylic acid,[24] testosterone-17-hemisuccinate,[6] monosuccinyl ecdysterone,[25] 3-*O*-succinyldigitoxigenin,[26] cholic acid,[27] thyroxine,[28] prostaglandins,[29] synthetic estrogens,[30] clonazepam-3-hemisuccinate,[31] and reserpine[32] are among the compounds linked in this way.

Carbodiimides

This is another direct method that has been used extensively in preparing conjugated antigens. Uridine 5′-carboxylic acid was coupled to a mul-

[23] J. R. Vaughan, Jr. and R. L. Osato, *J. Am. Chem. Soc.* **74**, 676 (1952).
[24] M. H. Karol and S. W. Tanenbaum, *Proc. Natl. Acad. Sci. U.S.A.* **57**, 713 (1967).
[25] M. L. deRiggi, M. H. Hirn, and M. A. Delaage. *Biochem. Biophys. Res. Commun.* **66**, 1307 (1975).
[26] G. C. Oliver, Jr., B. M. Parker, D. L. Brasfield, and C. W. Parker, *J. Clin. Invest.* **47**, 1035 (1968).
[27] G. J. Beckett, N. M. Hunter, and W. P.-R. Iain, *Clin. Chim. Acta* **88**, 257 (1978).
[28] W. H. Churchill and D. F. Tapley, *Nature (London)* **202**, 29 (1964).
[29] B. M. Jaffe, J. W. Smith, W. T. Newton, and C. W. Parker, *Science* **171**, 494 (1971).
[30] R. J. Warren and K. Fotherby, *J. Endocrinol.* **62**, 605 (1974).
[31] W. R. Dixon, R. L. Young, R. Ning, and A. J. Liebman, *J. Pharm. Sci.* **66**, 235 (1977).
[32] A. Levy, K. Kawashima, and S. Spector, *Life Sci.* **19**, 1421 (1976).

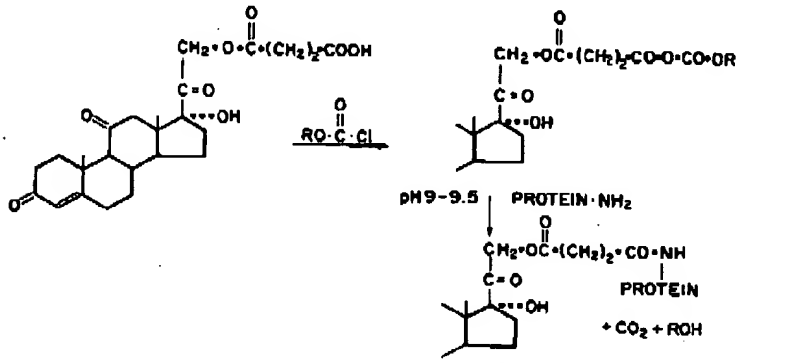
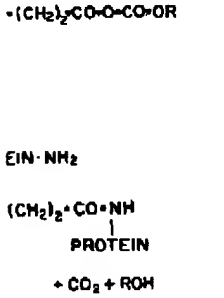


FIG. 2. Preparation of cortisone–21-hemisuccinate conjugate by mixed anhydride procedures.

tichain polypeptide, poly(DL-alanyl)-poly(L-lysine) with dicyclohexylcarbodiimide in a 95% dimethylformamide medium as solvent.[33] Coupling reactions can be carried out in aqueous solution by use of the water-soluble carbodiimides, such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide · HCl or 1-cyclohexyl-3-[2-morpholinyl-(4)-ethyl]carbodiimide metho-*p*-toluenesulfonate,[34,35] both commercially available reagents. Angiotensin and bradykinin, both small polypeptides (MW approximately 1000), were coupled to proteins in the first utilization of this procedure.[36] The authors believed that the reaction was between the N-terminal amino group of the peptide and the protein, but provided no evidence for this. On the other hand, they later used similar techniques to couple angiotensin to polylysine with the water-soluble reagent *N*-ethylbenzisoxazole,[37] a reaction that is possible only if the carboxyl group of angiotensin participates. One case in which it was definitely established that carbodiimides activate the carboxyl group of the peptide relates to the production of antibody to gastrin tetrapeptide.[11] In this case, the amino end of the peptide was blocked by a *tert*-butyloxycarbonyl (*t*-BOC) group. The *t*-BOC group was subsequently removed with trifluoroacetic acid. Carbodiimides were also used by Dietrich[38] and by Haber *et al.*[39] Additional components of

[33] M. Sela, H. Ungar-Waron, and Y. Schechter, *Proc. Natl. Acad. Sci. U.S.A.* **52**, 285 (1964).
[34] J. C. Sheehan, P. A. Cruickshank, and G. L. Boshart, *J. Org. Chem.* **26**, 2525 (1961).
[35] J. C. Sheehan and J. S. Hlavka, *J. Org. Chem.* **21**, 439 (1956).
[36] T. L. Goodfriend, L. Levine, and G. Fasman, *Science* **143**, 1344 (1964).
[37] T. L. Goodfriend, G. Fasman, D. Kemp, and L. Levine, *Immunochemistry* **3**, 223 (1966).
[38] F. M. Dietrich, *Immunochemistry* **4**, 65 (1967).
[39] E. Haber, L. B. Page, and G. A. Jacoby, *Biochemistry* **4**, 693 (1965).



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biological interest that were coupled to carriers with water-soluble carbodiimides include gastrin,[40] adenosine 3′,5′-cyclic phosphate,[41] morphine,[42] lysergic acid diethylamide,[43] and prostaglandin.[44,45] In three cases,[42–44] the carrier used was polylysine and immunization was done with a complex of the conjugate and succinylated hemocyanin. This improved technique minimized the extent of the immunological response to the carrier portion of the immunogen.

Tobramycin,[46] 1-β-D-arabinofuranosylcytosine,[47] cocaine metabolites,[48] prednisone-21-hemisuccinate,[49] synthetic narcotic analgesic drugs,[50] DL-methadol-hemisuccinate,[10] ochratoxin A,[51] digitoxigenin,[26] and gentamicin[52] are among additional compounds linked to proteins by means of carbodiimides. Digitoxigenin[26] was linked to a carrier protein, by both the carbodiimide and the mixed anhydride procedures, in the same laboratory. The mixed anhydride yielded a conjugate with 13 haptenic groups compared with 5 groups via the carbodiimide procedure.

Specific antibodies to α-melanotropin were obtained with carbodiimide using the peptide with its internal lysine blocked by an ε-methylsulfonylethoxycarbonyl group. Linkage to the amino groups of a protein then occured via the single glutamic acid residue remaining in the peptide, after which the protecting group was removed by 2 *N* Na_2CO_3.[53]

If the haptenic molecule is not soluble in water, it can be dissolved in water-miscible solvents, such as dimethylformamide, and added to the aqueous protein solution.[45]

[40] J. D. Young, D. J. Byrnes, D. J. Chisholm, F. B. Griffiths, and L. Lazarus, *J. Nucl. Med.* **10**, 746 (1969).
[41] A. L. Steiner, D. M. Kipnis, R. Utiger, and C. Parker, *Proc. Natl. Acad. Sci. U.S.A.* **64**, 367 (1969).
[42] H. Van Vunakis, S. Wasserman, and L. Levine, *J. Pharmacol. Exp. Ther.* **180**, 514 (1972).
[43] H. Van Vunakis, J. T. Farrow, H. B. Gjika, and L. Levine, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1483 (1971).
[44] L. Levine and H. Van Vunakis, *Biochem. Biophys. Res. Commun.* **41**, 1171 (1970).
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[46] A. Broughton, J. B. Strong, L. K. Pickering, and G. P. Brodey, *Antimicrob. Agents Chemother.* **10**, 652 (1976).
[47] T. Okabayashi, S. Mihara, D. B. Repke, and J. G. Moffat, *Cancer. Res.* **37**, 619 (1977).
[48] B. Kaul, S. J. Millian, and B. J. Davidow, *Pharmacol. Exp. Ther.* **199**, 171 (1976).
[49] A. W. Melkle, J. A. Weed, and F. H. J. Tyler, *J. Clin. Endocrinol. Metab.* **41**, 717 (1975).
[50] H. Van Vunakis, D. S. Freeman, and H. B. Gjika, *Res. Commun. Chem. Pathol. Pharmacol.* **2**, 379 (1975).
[51] O. Aalund, K. Brunfeldt, B. Hald, P. Krogh, and K. Poulsen, *Acta Pathol. Microbiol. Scand. C* **83**, 390 (1975).
[52] J. E. Lewis, J. C. Nelson, and H. A. Elder, *Nature (London)* **94**, 214 (1972).
[53] H. G. Kopp, A. Eberle, P. Vitius, W. Lichensteiger, and R. Schwyzer, *Eur. J. Biochem.* **75**, 417 (1977).

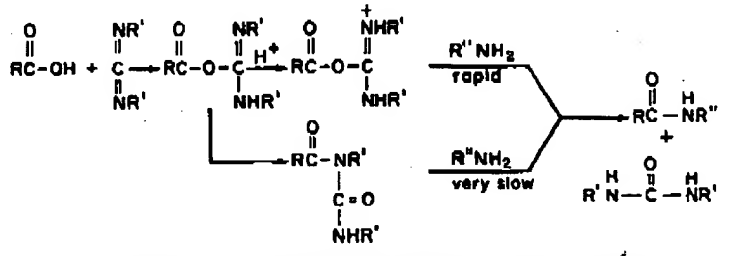

FIG. 3. Mechanism of carbodiimide-mediated preparation of amides.

The conditions of the reaction are very simple. The carrier, an excess of hapten and the reagent are simply stirred together in an aqueous solution for 30 min to several days depending upon the procedure. The reaction is followed by dialysis, and the product is isolated by lyophilization. The reaction mechanism is as shown in Fig. 3. There are two possible pathways, the desired one being catalyzed by H^+. The protein carrier, however, is most reactive at higher pH, where dissociation of the lysine ammonium groups occurs. A compromise is therefore necessary to provide the most favorable conditions; a pH near 6 is usually chosen. In our experience, the use of water-soluble carbodiimides has not always been successful. On occasion, extensive alteration of the carrier has occurred with little if any substitution by haptenic groups. It is possible to be led astray because antibody is produced to the altered protein, and this antibody does not react with the protein in its original state. Nevertheless, it is a generally efficacious method of preparing conjugates.

It is of interest that water-soluble carbodiimides have been used to couple nucleotides directly to proteins, presumably by formation of a P-N bond.[54-58]

Miscellaneous Carboxyl Methods

An aspirin–protein conjugate was prepared by first converting aspirin (acetylsalicylic acid) to the acetylsalicylazide.[59] The azide was coupled to rabbit serum globulin in a 1:1 dioxane–water solution maintained alkaline to phenolphthalein by the addition of base. About 25 to 35 haptenic

[54] M. J. Halloran and C. W. Parker, *J. Immunol.* 96, 373 (1966).
[55] M. J. Halloran and C. W. Parker, *J. Immunol.* 96, 379 (1966).
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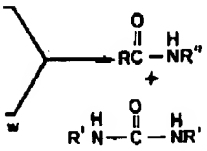
[57] S. A. Khan, M. Z. Humayun, and T. M. Jacob, *Nucl. Acids Res.* 4, 2997 (1977).
[58] S. A. Khan and T. M. Jacob, *Nucl. Acids Res.* 4, 3007 (1977).
[59] G. C. Butler, C. R. Harington, and M. E. Yuill, *Biochem. J.* 34, 838 (1940).



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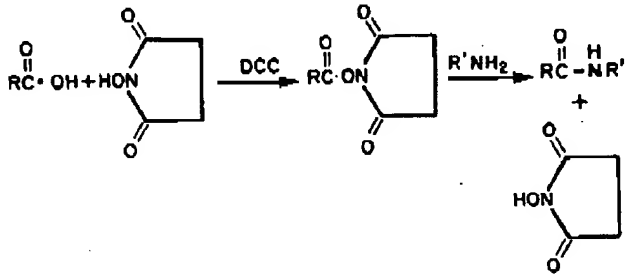

FIG. 4. Preparation of amides using *N*-hydroxysuccinimide. DCC, dicyclohexylcarbodiimide.

groups were conjugated per molecule of globulin. A similar procedure was used for thyroxine.[60] Antibodies specific for thyroxine have also been obtained by using, as antigen, tetraiodothyropropionic acid coupled to protein by the mixed anhydride method.[26]

The conversion of aspirin to an acid chloride that can react directly with protein was also reported.[61]

The insect juvenile hormone DL-10,11-epoxyfarnesoic acid was coupled to protein by a procedure that should find extensive use.[62,63] We had been unable to effect the reaction with water-soluble carbodiimides, obtaining only unsubstituted, altered protein (see above). The N-hydroxysuccinimide ester was prepared by reaction of the juvenile hormone with *N*-hydroxysuccinimide (commercially available) in the presence of dicyclohexylcarbodiimide (Fig 4). *N*-Hydroxysuccinimide esters are used in peptide synthesis.[64] They are quite stable if kept dry but react quickly and in good yield with amino groups to form amide or peptide bonds. Conjugates containing 20 juvenile hormone groups were used to raise specific antibodies in rabbits. Antibodies to ecdysone were made similarly.[65]

Carbonyldiimidazole is another commercially available reagent that has been used to link haptens to proteins by means of amide bonds, for example in the preparation of a BSA conjugate of fluoxymesterone.[66]

[60] R. F. Clutton, C. R. Harrington, and M. E. Yuill, *Biochem. J.* 32, 1119 (1938).
[61] L. M. Weiner, M. Rosenblatt, and H. Howes, *J. Immunol.* 90, 788 (1963).
[62] R. C. Lauer, P. Soloman, K. Nakanishi, and B. F. Erlanger, *Fed. Proc.* 32, 500 (1972).
[63] R. C. Lauer, P. H. Soloman, K. Nakanishi, and B. F. Erlanger, *Experientia* 30, 558 (1974).
[64] G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Am. Chem. Soc.* 86, 1839 (1964).
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[66] W. A. Colburn, *Steroids* 25, 43 (1975).

Haptens with Amino Groups

Two classes of haptens with available amino groups must be considered—the aromatic amines and the aliphatic amines.

Aromatic Amines

Much of Landsteiner's pioneer work[1] was carried out with haptens that were aromatic amines. The compounds were converted to diazonium salts with nitrous acid and allowed to react with proteins at alkaline pH (approximately 9). Reaction occurred primarily with histidine, tyrosine, and tryptophan residues of the protein carrier. For a representative procedure, see Kabat[67] (p. 799 *et seq.*). An interesting application of this procedure was the preparation of a chloramphenicol–protein conjugate which was used to elicit antibodies specific for chloramphenicol.[68] In this case, a prior reduction of the nitro group of chloramphenicol to an amino group was required. As early as 1937, carcinogenic compounds were conjugated to protein carriers by means of their isocyanate derivatives which were prepared from amines.[69] Immune sera were raised, and their properties were studied.[69,70]

Aliphatic Amines

Aliphatic amines can be caused to react with proteins by using water-soluble carbodiimides. Examples include bradykinin and angiotensin,[36] tobramycin,[46] gentamicin,[53] adriamycin,[71] 5-hydroxytryptamine (serotonin),[72] cortisol-21-amine,[73] and spermidine.[74]

Aliphatic amines can be converted to a *p*-nitrobenzoylamide by reaction with *p*-nitrobenzoyl chloride. The amide derivative can then be reduced to a *p*-aminobenzoyl derivative which can be coupled to proteins by diazotization, as described above. Among the haptens conjugated this

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[68] R. N. Hamburger. *Science* **152**, 203 (1966).
[69] H. J. Creech, *Cancer Res.* **12**, 557 (1952).
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[71] Y. -H. Chien and L. Levine. *Immunochemistry* **12**, 291 (1975).
[72] B. Peskar and S. Spector, *Science* **179**, 1340 (1973).
[73] Y. Kobayashi, T. Ogihara, K. Amitani, F. Watanabe. T. Kigushi, I. Ninomiya, and Y. Kumahara, *Steroids* **32**, 137 (1978).
[74] F. Bartos, D. Bartos, A. M. Dolney, D. P. Grettie, and R. A. Campbell, *Res. Commun. Chem. Pathol. Pharmacol.* **19**, 295 (1978).

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A. Campbell, *Res. Commun.*

way are a series of C-terminal peptide sequences of the tobacco mosaic virus protein[75–77] and angiotensin.[78,79]

Angiotensin has also been attached by its N-terminal amino group to the amino groups of a carrier by means of the bifunctional reagent *m*-xylylene diisocyanate.[39] Tolylene 2,4-diisocyanate has been used in a similar manner to prepare bradykinin conjugates.[80] Haptens containing amino groups have also been covalently linked to amino groups of protein carriers with glutaraldehyde. Among the haptenic groups conjugated in this manner are adrenocorticotropic hormone (ACTH),[81] glucagon,[82] and normetanephrine.[83]

A novel procedure has been used to link nortryptyline to BSA.[84] Its aliphatic secondary amine was converted to a succinamic acid derivative that was caused to react with the protein by using a water-soluble carbodiimide. The antibody was used to assay for various tricyclic antidepressants, including imipramine. In another novel procedure involving nortryptyline, the secondary amine was allowed to react with *N*-(4-bromobutyl)phthalimide. After removal of the phthalimido group, the resulting primary aliphatic amine was caused to react with carboxyl groups on BSA by using a water-soluble carbodiimide.[85,86]

Haptens with Available Hydroxyl Groups

This class of haptens includes alcohols, phenols, sugars, polysaccharides, and nucleosides. In most cases, derivatives of this class of compounds must be made in order to introduce functional groups capable of reacting with proteins.

Hemisuccinates

A simple procedure, first introduced in our work with steroid–protein conjugates, is the conversion of the alcohol to the half ester of succinic

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[74] F. A. Anderer and H. D. Schlumberger, *Biochim. Biophys. Acta* 115, 222 (1966).
[77] F. A. Anderer and H. D. Schlumberger, *Biochim. Biophys. Acta* 97, 503 (1965).
[78] S. D. Deodhar, *J. Exp. Med.* 111, 419 (1960).
[79] S. D. Deodhar, *J. Exp. Med.* 111, 429 (1960).
[80] J. Spragg, K. F. Austen, and E. Haber, *J. Immunol.* 96, 865 (1966).
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[83] B. A. Peskar, B. M. Peskar, and L. Levine, *Eur. J. Biochem.* 26, 191 (1972).
[84] D. J. Brunswick, B. Needleman, and J. Mendels, *Life Sci.* 22, 137 (1978).
[85] G. W. Aherne, E. M. Piall, and V. Marks, *Br. J. Clin. Pharmacol.* 3, 561 (1976).
[86] K. P. Maguire, G. D. Burrows, T. R. Norman, and B. A. Scoggins, *Clin. Chem.* 24, 549 (1978).

acid (i.e., the hemisuccinate). The hemisuccinate has an available carboxyl group that can be made to react by any of the procedures described above. Conversion to the hemisuccinate requires a reaction with succinic anhydride in pyridine. A representative procedure can be found in the papers on steroid–protein conjugates.[45] Another example is the preparation of the hemisuccinate of cyclic adenosine monophosphate (cAMP).[41] More recently, in an excellent study of the specificity of antiestrogen antibodies.[87] 11α-hydroxyhemisuccinates of estrone and estradiol were prepared and linked to BSA by the mixed anhydride technique. Other examples include hemisuccinates of β-dl-methadol,[10] 3-hydroxyclonazepam,[31] ecdysterone,[25] propanolol,[88] Δ^9-tetrahydrocannabinol,[89] and 1-β-D-arabinofuranosylcytosine.[47]

Chlorocarbonates

Another alternative is the reaction of the determinant group with an equimolar quantity of phosgene to yield the highly reactive chlorocarbonate, which reacts directly with the amino groups of the protein in the presence of bicarbonate. An example is the conversion of testosterone to testosterone-17-chlorocarbonate.[4]

Aminophenyl Derivatives

Phenols can be converted to active reagents by reaction with diazotized *p*-aminobenzoic acid. In this way, a carboxyl group is introduced into the molecule. This type of reaction was carried out successfully with 17β-estradiol.[90] More recently, a similar procedure was used to make conjugates of Δ^7-tetrahydrocannabinol[89] and reserpine.[92]

The classical procedure for the coupling of sugars to proteins involves the formation of *p*-nitrophenylglycosides, the conversion of the latter by hydrogenation to *p*-aminophenylglycoside, and then attachment to the protein by diazotization. This method was used by Landsteiner[1] for a number of preparations. A variant of this method, used by Goebel[91,92] and Goebel and Hotchkiss,[93] was conversion to the aminobenzyl ether followed by diazotization.

[87] F. C. den Hollander, B. K. van Weemen, and G. F. Woods, *Steroids* 23, 549 (1974).
[88] K. Kawashima, A. Levy, and S. Spector, *J. Pharmacol. Exp. Ther.* 196, 517 (1976).
[89] P. T. Tsui, K. A. Kelly, M. M. Ponpipon, and A. H. Sehon, *Can. J. Biochem.* 52, 252 (1974).
[90] N. Weliky and H. H. Weetall, *Immunochemistry* 2, 293 (1965).
[91] W. F. Goebel, *J. Exp. Med.* 64, 29 (1936).
[92] W. F. Goebel, *J. Exp. Med.* 68, 469 (1938).
[93] W. F. Goebel and R. D. Hotchkiss, *J. Exp. Med.* 66, 191 (1937).

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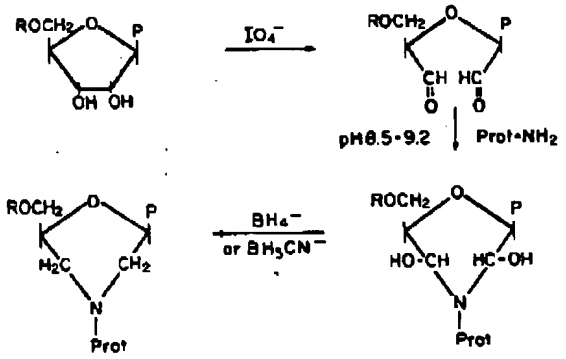

P = Purine or Pyrimidine
R = H, $-PO_3H_2$ or 3'-nucleotide
Prot-NH_2 = Carrier protein, NH_2 groups

FIG. 5. Preparation of conjugates using periodate procedure.

Oxidation to Dialdehydes

A relatively simple procedure developed for the preparation of nucleoside– and nucleotide–protein conjugate[94–98] makes use of the reaction of vicinal hydroxyl groups with periodate to yield dialdehydes (Fig. 5). The dialdehydes, without isolation, are caused to react with the amino groups of protein at pH 9.5 in aqueous solution to yield aldimines, which are stabilized by reduction with sodium borohydride. Only the final conjugate is isolated in this procedure, which is simple to run and yields conjugates with as many as 30 determinant groups per molecule of albumin. It should be applicable to all compounds with vicinal hydroxyl groups, such as glycols, glycerol derivatives, and glycosides, and has been used successfully for the preparation of digoxin–protein conjugates[99] and ouabain.[100] Modi-

[94] B. F. Erlanger and S. M. Beiser, *Proc. Natl. Acad. Sci. U.S.A.* **52**, 68 (1964).
[95] B. F. Erlanger, D. Senitzer, O. J. Miller, and S. M. Beiser, *Acta Endocrinol. (Copenhagen) Suppl.* **168**, 206 (1972).
[96] R. M. D'Alisa and B. F. Erlanger, *Biochemistry* **13**, 3575 (1974).
[97] R. M. D'Alisa and B. F. Erlanger, *J. Immunol.* **116**, 1629 (1976).
[98] S. M. Beiser, S. W. Tanenbaum, and B. F. Erlanger, this series, Vol. 12B, p. 889.
[99] V. P. Butler and J. P. Chen, *Proc. Natl. Acad. Sci. U.S.A.* **57**, 71 (1967).
[100] T. W. Smith, *J. Clin. Invest.* **51**, 1583 (1972).

fications have been made to prepare conjugates with alkaline-sensitive nucleosides and nucleotides.[101-103]

In a recent procedure to detect carcinogen-DNA adducts by radioimmunoassay,[104] *N*-(guanosin-8-yl)acetylaminofluorine was linked to BSA by the periodate method, and the conjugate was used to elicit specific antibodies to this product, which is formed when *N*-acetoxy-2-acetylaminofluorene reacts with DNA.

Oxidation to Carboxyl

Oxidation of the 5'-hydroxyl groups of uridine,[24,105] pseudouridine,[24] and other nucleosides[33] has made it possible to conjugate these compounds to proteins by methods amenable for the reaction of carboxylic acid derivatives with proteins.

Miscellaneous Hydroxyl Methods

Another method of seemingly general applicability to carbohydrates was used by Coat *et al.*[106] to conjugate uridine to proteins. The isopropylidine derivative was allowed to react with *p*-nitrobenzoyl chloride to yield the 5' ester. Removal of the isopropylidine protecting group and hydrogenation of the nitro group made it possible to link the uridine derivative to the protein by a diazotization reaction.

Some rather novel chemistry was described in two recent very interesting papers describing a radioimmunoassay procedure for ADP-ribose.[107] Adenine-N^6-carboxymethylated NAD was prepared and converted to N^6-carboxymethylated ADP-ribose by NAD glycohydrolase. N^6-Carboxymethylated ADP-ribose was then linked to BSA using water-soluble carbodiimide.

The bifunctional reagent sebacoyl dichloride has been used to convert alcohols to acid chlorides, which, at pH 8.5, react readily with proteins. This procedure was used by Bailey and Butler[108] to prepare a cholesterol–protein conjugate.

[101] L. Rainen and B. D. Stollar, *Nucl. Acids Res.* 4, 4877 (1978).
[102] R. D. Meredith and E. F. Erlanger, *Fed. Proc.* 37 (6), 1503 (1978).
[103] J. Wollack, *Fed. Proc.* 37 (6), 1632 (1978).
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[105] M. Sela and H. Ungar-Waron, *Fed. Proc.* 24, 1438 (1965).
[106] J. P. Coat, S. David, and J. C. Fischer, *Bull. Soc. Chim. Fr.* 21, 2489 (1963).
[107] R. Bredehorst, A. M. Ferro, and H. Hilz, *Eur. J. Biochem.* 82, 105 (1978).
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Haptens with Carbonyl Groups

Ketones and aldehydes can be used as haptenic determinant groups by converting them to *O*-(carboxymethyl) oximes. This is done by reacting them with *O*-(carboxymethyl)hydroxylamine (NH_2OCH_2COOH, sold commercially as carboxylmethoxylamine or aminooxyacetic acid). This serves to introduce a carboxyl group, which is exploited as described above. Examples of this methodology can be found in the coupling of testosterone-3-(*O*-carboxymethyl) oxime, estrone-17-(*O*-carboxymethyl) oxime, and progesterone-20-(carboxymethyl) oxime to bovine serum albumin with the mixed anhydride technique.[4,5] Prepared in a similar manner were the 3-(*O*-carboxymethyl) oxime derivative of medroxyprogesterone acetate,[109] the 3-carboxymethyl oxime of aldosterone-18-21-diacetate.[110] Similarly, *O*-carboxymethyl derivatives were prepared from the synthetic progestogens norethisterone and norgestrel.[111]

5α-Dihydrotestosterone-11-(*O*-carboxymethyl) oxime was synthesized[112] in an elegant multistep procedure that included a microbiological reduction and a selective hydrolysis of a dioxime. The final product was prepared by reaction of 17β-hydroxy-5α-androstane-3, 11-dione-11-oxime with sodium chloroacetate to give the *O*-carboxymethyl oxime.

The ketone groups of aldosterone, corticosterone, and cortisol were derivatized with *p*-hydrazinobenzoic acid.[113] The resulting carboxylic acid derivatives could be linked to BSA with water-soluble carbodiimide. Aldehydes can be conjugated to proteins directly by Schiff base formation followed by stabilization of the bond by reduction with sodium borohydride. Pyridoxal and pyridoxal phosphate are examples of haptens conjugated in this manner.[114,115]

Other Reactions

Penicillenic acid was conjugated to protein by an interesting procedure that included modification of the protein carrier.[116] Penicillenic acid has a reactive sulfhydryl group capable of forming disulfide bonds with other

[109] M. Hiroi, F. Z. Stanczyk, U. Goebelsmann, P. F. Brenner, M. E. Lumkin, and D. R. Mishele, Jr., *Steroids* 26, 373 (1975).
[110] C. A. Bizoleon, J. -F. Riviere, P. Franchimont, A. Faure, and B. Claustrat, *Steroids* 23, 809 (1974).
[111] R. J. Warren and K. Fotherby, *J. Endocrinol.* 62, 605 (1974).
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[113] B. Africa and E. Huber, *Immunochemistry* 8, 479 (1971).
[114] F. Cordoba, C. Gonzalez, and P. Rivera, *Biochim. Biophys. Acta* 127, 151 (1966).
[115] R. Ungar-Waron and M. Sela, *Biochim. Biophys. Acta* 124, 147 (1966).
[116] A. L. deWeck and H. N. Eisen, *J. Exp. Med.* 112, 1227 (1960).

sulfhydryl groups. The carrier proteins (e.g., human γ-globulin or bovine γ-globulin) were artificially enriched in sulfhydryl groups by reaction with *N*-acetylhomocysteine thiolactone.[117] The coupling reaction with an excess of penicillenic acid was then carried out in acetate buffer at pH 4 in the presence of H_2O_2.

Antibody to progesterone was also obtained by immunization with conjugates prepared from thiolated proteins.[118] Bovine serum albumin was thiolated by reaction with *S*-acetylmercaptosuccinic anhydride. After deacetylation, coupling was achieved with 6β-bromoprogesterone.

Bis-diazotized benzidine can be used as a bridging reagent between proteins and haptens containing aromatic groups that react with diazonium compounds. A conjugate of thyrotropin-releasing hormone (which contains a reactive histidine residue) was obtained in this way.[119]

A novel approach has been to allow serotonin to react with protein via the Mannich reaction.[120] This is a simple reaction that enables one to use formaldehyde as a bridge between the amino groups of a protein and compounds containing one or more reactive hydrogens. The Mannich reaction has also been used to prepare reserpine conjugates.[82] The antibody titers were not as satisfactory as those elicited by conjugates prepared by a *p*-carboxyazobenzene derivative linked to BSA by the mixed anhydride procedure.

Among the low molecular weight haptens that have been used as determinant groups are substances that are reactive enough to be coupled to proteins directly. Dinitrofluorobenzene has been used to prepare antigens for the stimulation of antidinitrophenyl antibodies. These have been very useful in studies of the binding characteristics and structure of immunoglobulins. Antibodies that react with deoxyribonucleic acid (DNA) have been elicited by immunization with the product of the reaction of 6-trichloromethylpurine[121–123] with BSA. Nucleotide protein conjugates have also been made by using carbodiimide to link the nucleotide to the protein.[56–58] Antipenicillin antibodies have been produced by immunization

[117] R. Benesch and R. E. Benesch, *in* "Sulfur in Proteins" (R. Benesch, R. E. Benesch, P. D. Boyer, I. M. Klotz, W. R. Middlebrook, A. G. Szent-Györgyi, and D. R. Schwartz, eds.), pp. 15–24. Academic Press, New York, 1959.
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[120] N. S. Ranadive and A. H. Sehon, *Can. J. Biochem.* 45, 1701 (1967).
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[122] S. Cohen, E. Thom, and A. Bendich, *J. Org. Chem.* 27, 3545 (1962).
[123] V. P. Butler, Jr., S. M. Beiser, B. F. Erlanger, S. W. Tanenbaum, S. Cohen, and A. Bendich, *Proc. Natl. Acad. Sci. U.S.A.* 48, 1597 (1962).

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progesterone.
ng reagent between
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ng hormone (which
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The antibody titers
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e been used as de-
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with penicillin–protein conjugates. The latter were prepared by the reaction of penicillin with protein under slightly alkaline conditions.[124–126]

A tetrahydrocannabinol–BSA conjugate has been made by reaction with 10-iodotetrahydrocannabinol-9-isocyanate.[89] L-Phenylalanine mustard is another example of a reactive hapten.[127]

Characterization of the Conjugates

Generally, the haptenic group has an absorbance spectrum that can allow one to differentiate it from the protein carrier. This is particularly true for azo derivatives, which absorb in the visible range. However, even if there is overlap in the two spectra, reasonably accurate determinations of the number of haptenic molecules per carrier protein can be determined from difference spectra.

A more convenient and direct procedure, introduced by Abraham *et al.*,[128] is the incorporation of some radioactive hapten in the conjugation procedure. A direct estimation of the extent of substitution can be made by counting undialyzable radioactive material.

A procedure introduced by us for steroid–protein conjugates[4] was the estimation of the remaining free amino groups with the dinitrophenylation technique of Sanger.[129] ε-Dinitrophenyllysine was not isolated but was estimated directly by spectrophotometry after ether extraction of the acid hydrolyzate. A control with unsubstituted carrier was always run concomitantly, and the difference between the two was taken to be the extent of substitution by hapten.

We have also used a procedure of Habeeb[130] in which trinitrobenzene sulfonic acid is used as the reagent for estimation of free amino groups in the conjugate. Spectrophotometric comparison of the intact protein conjugate and the original carrier protein is possible: no acid hydrolysis is required. We found this procedure to be convenient and entirely satisfactory, an example being the case of insect juvenile hormone–protein conjugates.[62,63,65]

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General Comments

It was not the purpose of this review to present a comprehensive survey of hapten–protein conjugates, but rather to provide sufficient information to guide the researcher in the design of his or her particular experiments. On the other hand, the most practical approaches to the preparation of hapten–protein conjugates were cited.

Many of the methods used to prepare immunogenic conjugates have also been used to link drugs to carrier molecules (including antibodies) in order to "target" cytotoxic drugs. Two reviews that are useful in that they describe many of the methods used to make the carrier–drug conjugates are those by Trouet[131] and by Ghose.[132] The information in these reviews should be useful to immunologists as well.

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[5] Production of Reagent Antibodies

By B. A. L. Hurn and Shireen M. Chantler

Immunization

The explosion of interest in immunoassay procedures during the last two decades has resulted in an enormous volume of literature describing, for the most part, satisfactory results of immunization. During the same period, knowledge of the underlying mechanisms of the immune response has advanced greatly from an original state of almost total ignorance. Perhaps unfortunately, those who have pursued basic understanding have seldom been much concerned with the practical problems of making useful reagent antibodies. As a result, with few exceptions the literature of immunization does no more than describe successful procedures, and the variety of these is legion. In the usual way of things, abortive attempts are seldom mentioned, let alone described, yet anyone with practical experience who has also discussed the matter with colleagues will be well aware that all the successful methods have also, at other times or in other places, singularly failed to give the desired results. Not surprisingly, the failure rate is higher when making antisera for more demanding test systems, such as radioimmunoassay, than for immunoprecipitin methods, for instance. Much of the uncertainty over the outcome of immunization may be ascribed to variations in individual animal response; however, when an

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